Supplementary information

Human BRCA2 protein promotes RAD51 filament formation on RPA-covered ssDNA

Jie Liu[1], Tammy Doty[1,3], Bryan Gibson[1,3], and Wolf-Dietrich Heyer [1,2#]

[1] Departments of Microbiology and [2] of Molecular and Cellular Biology,

University of California, Davis, Davis, CA 95616

[3] Present address: Novozymes, Inc., Davis CA 95618, USA (TD); Department of Molecular Biology and Genetics, Cornell University, Ithaca NY 14853, USA (BG).

Corresponding author: Tel. (530) 752-3001 FAX (530) 752-3001 Email wdheyer@ucdavis.edu

Supplementary Methods

Supplementary Figure

Supplementary References

Supplementary Methods

GST-BRCA2-FLAG-His10 plasmid construction. The human *BRCA2* cDNA was kindly provided by Myriad Genetics in a vector that allowed release of the gene by *Sal*I restriction [1]. Vector pJN58 [2] containing the *GST* coding sequence under control of a *GAL1* promoter was modified by site directed mutagenesis to include a *Sal*I restriction site. After insertion of the *BRCA2* gene into the modified pJN58 vector, PCR was used to amplify the 3' end of the gene beginning with an *Sph*I restriction site, adding FLAG and His10 tags, and a *Xho*I restriction site. The amplified fragment was then cloned back into the plasmid between the *Sph*I and *Sal*I sites. The DNA sequence of the entire construct (pWDH588) was verified. The DNA construct translates into a GST-BRCA2-FLAG-His10 protein of 413.3 kDa, with twelve amino acids (SLVPRGSGSTAT) between the GST tag and BRCA2, and one amino acid (D) between BRCA2 and the FLAG-$(His)_{10}$ tag.

DSS1 plasmid constructions. The human *DSS1* sequence (NCBI NM_006304 [3]) was reconstructed from 6 oligonucleotides (sequences available upon request) and cloned into pYESTRP (Invitrogen) using 5′ *Bam*HI and 3′ *Eco*RI sites resulting in plasmid pWDH754. The entire coding sequence was verified by DNA sequencing. The *Bam*HI – *Eco*RI fragment was cloned into pTYB11 to generate pWDH755, where DSS1 is fused to the intein coding sequence, resulting in a self-splicing fusion protein for expression in *E. coli* and purification of authentic human DSS1 protein [4].

Purification of human RAD51 and RPA: Human Rad51 and RPA were purified exactly as described [5,6].

Purification of GST-BRCA2-FLAG-His10 protein. The *S. cerevisiae* strain WDHY668 (*MATa/MATα ura3-52/ura3-52 trp1/trp1 leu2Δ1/leu2Δ1 his3Δ200/ his3Δ200 pep4::HIS3/ pep4::HIS3 prb1Δ1.6R/ prb1Δ1.6R can1/can1 GAL/GAL*) containing plasmids pWDH588 and pWDH754 was inoculated into a 45 L culture with basic media –ura and –trp (0.17 % (w/v) yeast nitrogen base, 0.5 % (w/v) sodium lactate, 3 % (v/v) glycerol, 0.87 g/l amino acid mix without uracil and without tryptophan) and grown at 30 °C. BRCA2 expression was induced by addition of 2 % (w/v) galactose when the cells reached OD_{600} of ≈ 2.0 and grown at 25 °C. There was no evidence for DSS1 expression in these cells. The cells were harvested after 13 hours by centrifugation and stored at -80 °C. The cells were resuspended in buffer A (20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 % glycerol, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin A, 2 mM benzamidin, 1 μM leupeptin) containing 1 M NaCl and lysed with acid-washed glass beads (0.5 mm diameter). The cell extract was centrifuged at 40,000 rpm for 45 minutes at 4 °C. The supernatant was precipitated with ammonium sulfate to obtain a fraction from 20-45 % saturation. The pellet was dissolved in PBS buffer (2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.2)) containing 1 M NaCl. The suspension was applied to a 5 ml GSTrap FF (GE Healthcare) column. After washing, protein was eluted with buffer A (lacking DTT) containing 500 mM NaCl and 20 mM glutathione. Fractions containing GST-BRCA2 protein were pooled and applied to a column containing anti-FLAG M2 affinity gel (Sigma). Column was washed with 20 mM HEPES (pH 7), 500 mM NaCl and eluted with same buffer containing 100 μg/ml FLAG peptide. Fractions containing GST-BRCA2-FLAG-His10 (3-5 μg/preparation) were used for experiments described here. The gels shown in Fig. 1B were stained with Silver Stain Plus (BioRad). After protein purification, the *GST-BRCA2-FLAG-His10*

gene on the expression vector in the yeast overproduction strain was re-sequenced, establishing that no sequence changes occurred during culturing.

Purification of human DSS1 protein. E. coli ER2566 containing plasmid pWDH755 were grown at 37 °C until OD_{600} reached 0.6, then 0.5 mM (final concentration) IPTG was added to induce expression for 4 hrs at 25 °C. After harvesting, cells were sonicated in lysis buffer including 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 0.1 % Triton X-100 and the lysate was centrifuged at 14,000 rpm for 30 minutes at 4 °C. The supernatant was loaded onto a chitin column equilibrated with 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, and 0.1 % Triton X-100. After extensive washing, DSS1 was cleaved and eluted from the column using buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 50 mM DTT. Fractions were collected and loaded onto a Mono-Q column equilibrated with buffer B containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.2 mM EDTA, and 10 % glycerol. After wash, DSS1 was eluted using buffer B with a 100 – 1000 mM NaCl gradient. The fractions were pooled and dialyzed against the DSS1 storage buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 10 % glycerol. The final pool was aliquoted and flash frozen in liquid N_2 before storage at -80 °C. The gel shown in Figure 2e was stained with Denville Blue™ Protein Stain (Denville Scientific Inc.).

In vitro BRCA2-RAD51 physical interaction assay. 10 ng (0.1 nM) GST-BRCA2-FLAG-His10 were incubated with excess RAD51 (50 ng, 4.65 nM or 100 ng, 9.3 nM) at 22^0C for 1 hr in 250 µL binding buffer containing 25 mM Tris-HCl (pH 7.5), 4 mM $Mg(OAc)_2$, 50 mM NaCl, 1 mM DTT, 10% glycerol, and 0.05% Nonidet P-40. Equilibrated and BSA-treated Glutathione-

Sepharose 4B beads were added into the mixture and incubated for another hour. The beads and supernatant were separated by centrifugation and the beads were washed twice with 400 μL binding buffer. The pulled-down protein complexes were eluted by boiling at 95 °C for 3 min in 10 mL SDS-PAGE loading buffer and separated through a 4-12 % SDS-PAGE gradient gel. Separated proteins were then transferred onto nitrocellulose membrane for immunoblotting. GST-BRCA2-FLAG-His10 and GST control (GE Healthcare) proteins were detected using an anti-GST antibody (GE Healthcare) and human RAD51 with an anti-RAD51 antibody (Santa Cruz Biotech). The reactions contain 4.65 nM (50 ng) or 9.30 nM (100 ng) RAD51 and 0.1 nM (10 ng) or no BRCA2 protein. The pull-down efficiency of GST-tagged BRCA2 by Glutathione Sepharose beads was determined to be ~ 20 %. Either 1 mM AMP-PNP, 1 mM ADP, or 1 mM ATP with regenerating system (R.S.) consisting of 6 U/mL pyruvate kinase and 3 mM phosphoenolpyruvate, were used for the experiments in Figure 1d-f. The amount of purified BRCA2 was estimated based on silver-staining of SDS-PAGE gels. In the assay, 1/5th of the input BRCA2 was loaded directly onto SDS-PAGE gel with other pulldown samples as a protein standard to calculate the amount of BRCA2 pulled down. For RAD51, 1, 5, 10, or 20 ng of protein were loaded onto SDS-PAGE gel as protein standards to calculate the amount of RAD51 pulled down for each immunoblot. The immunoblot signals were quantified by densitometry and all experimental RAD51 values were within the linear range of the standard curve.

RAD51 binding to gapped DNA immobilized on magnetic beads: A gapped DNA substrate with 9 junctions was prepared by annealing ϕX174 circular ssDNA with four 80 nt oligonucleotides (oWDH802-805) and a 5′-biotinylated oligonucleotide (80 nts) similar to the design in reference [7]. The oligonucleotide sequences are available upon request. In the binding experiments, 150 μL slurry of magnetic beads containing 0.2 μM (nt concentration) ssDNA were incubated with 20

nM RPA and with the indicated amount of BRCA2 first in buffer containing 20 mM triethanolamine pH 7.5, 4 mM $Mg(OAc)_2$, 2 mM ATP or 1 mM ADP, 25 μg/mL BSA, 1 mM DTT, 5 % glycerol, 83 mM NaCl for 10 minutes at 22 °C. Then 13.3 nM RAD51 was added and incubated for 20 minutes. When Ca^{2+} and ATP were used, the buffer contained 3 mM $CaCl_2$ and 2 mM ATP. DSS1 (0.8, 8, 24 nM) was incubated with BRCA2 and RPA during the first incubation period. The beads were washed, and bound proteins were eluted and quantified by densitometry. Background protein binding was typically less than 3 %. The amount of bound proteins on gapped DNA substrates was compared with the protein standards on the same blot and quantified using densitometry, as described above.

Supplementary References

1 Wooster, R. *et al.* Identification of the breast cancer susceptibility gene BRCA2. *Nature* **378**, 789-791 (1995).

2 Nelson, J. R., Lawrence, C. W. & Hinkle, D. C. Thymine-thymine dimer bypass by yeast DNA polymerase zeta. *Science* **272**, 1646-1649 (1996).

3 Crackower, M. A. *et al.* Characterization of the split hand/split foot malformation locus SHFM1 at 7q21.3-q22.1 and analysis of a candidate gene for its expression during limb development. *Hum. Mol. Genet.* **5**, 571-579 (1996).

4 Chong, S. *et al.* Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. *Gene* **192**, 271-281 (1997).

5 Sigurdsson, S., Trujillo, K., Song, B. W., Stratton, S. & Sung, P. Basis for avid homologous DNA strand exchange by human Rad51 and RPA. *J. Biol. Chem.* **276**, 8798-8806 (2001).

6 Henricksen, L. A. & Wold, M. S. Replication protein A mutants lacking phosphorylation sites for p34(cdc2) kinase support DNA replication. *J. Biol. Chem.* **269**, 24203-24208 (1994).

7 Morimatsu, K. & Kowalczykowski, S. C. RecFOR proteins load RecA protein onto gapped DNA to accelerate DNA strand exchange: a universal step of recombinational repair. *Mol. Cell* **11**, 1337-1347 (2003).

Supplementary Figure 1

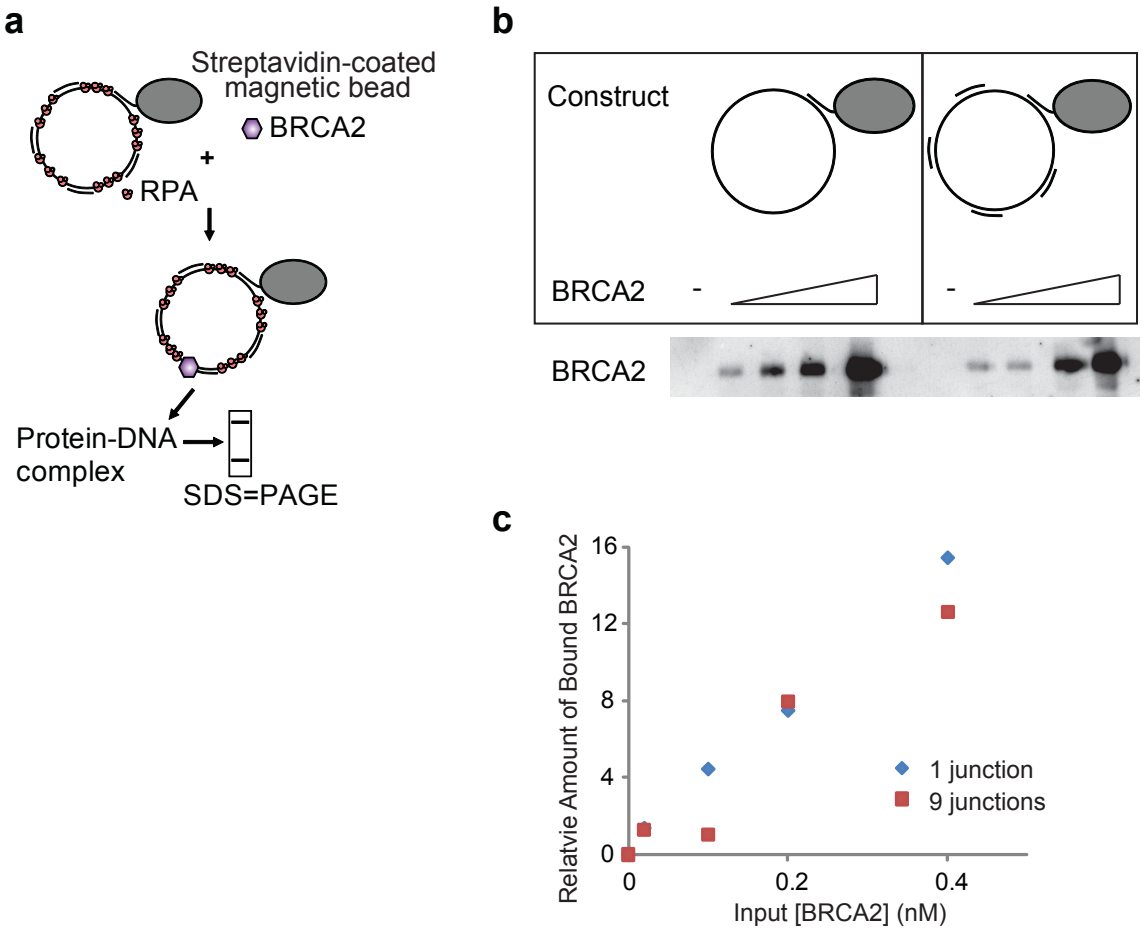

Figure S1. The amount of bound BRCA2 on RPA-covered gapped ssDNA does not correlate with the number of junctions in the DNA construct. a, Substrate and assay design for BRCA2 binding to RPA-covered gapped DNA. b, Immunoblot of BRCA2 bound to immobilized RPA-covered gapped DNA substrates with either one 5′-junction or nine junctions (five 5′- and four 3′-junctions). The reactions contained 0.2 μM (nt concentration) ssDNA, 20 nM RPA, and 0, 0.02, 0.1, 0.2, and 0.4 nM (0, 1.25, 6.25, 12.5, 25 ng) BRCA2.